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Nimbolide is the Principal Cytotoxic Component of Neem-Seed Insecticide Preparations

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Abstract: Several neem-seed extracts, some used for preparing commercial azadirachtin-containing insecticides, are cytotoxic to N1E-115 murine neuroblastoma cells with IC_{50} values of 20–200 μg extract ml^{-1} culture medium. Bioassay-directed fractionation by reversed-phase HPLC shows that the toxicity to N1E-115 cells is associated primarily with a single minor component identified by isolation and NMR and MS as nimbolide with an IC_{50} of 1·5 μg ml^{-1} (3·2 μM). The difference in quantity of nimbolide in seven neem extract sources generally correlates with their overall cytotoxicity. Three other limonoids (epoxyazadiradione, salannin and possibly deacetylsalannin) but not azadirachtin, nimbin and deacetylnimbin contribute in small part to the cytotoxicity. Reconstituted neem extract with only nimbolide removed is less cytotoxic than the original extract. It therefore appears that nimbolide is the principal cytotoxic component of the neem extracts examined and that such minor constituents may warrant consideration in safety evaluations.

Key words: azadirachtin, epoxyazadiradione, neem, nimbolide

1 INTRODUCTION

Seeds of the Indian neem tree, *Azadirachta indica* A. Juss (Meliaceae), are used for insecticide preparations registered for application to ornamentals and food crops. Neem-seed preparations are the most recently developed of the five botanical insecticides currently registered in the United States.[1] The active ingredients of pyrethrum and sabadilla act on the voltage-dependent sodium channel, of derris on respiratory metabolism, and of ryania on calcium-activated calcium channels; each of these targets is important in mammals as well as in insects. Neem, by contrast, acts slowly and more specifically as an insect antifeedant and growth regulator.[2–4] The primary insecticidal ingredient is azadirachtin (Fig. 1) which varies over a large range in concentration among the commercial neem-seed preparations. These extracts contain a variety of limonoids and other potentially bioactive compounds.[5] They are of low acute oral toxicity for laboratory mammals and are generally regarded as safe for use.[1] However, the neem-tree constituents are known to have many types of biological activity and as such they have been employed for centuries as medicinal products.[6]

In the course of a survey of botanicals for cytotoxicity we observed that certain neem-seed preparations showed relatively high activity on a broad spectrum of cell types. This report describes our bioassay-directed fractionation of these preparations leading to identification of nimbolide as the principal cytotoxic component.

2 MATERIALS AND METHODS

2.1 Chemicals

Seven neem-seed extracts examined were: A and B from W. R. Grace and Co. (Columbia, MD); C and D from N. W. Exports Ltd (Secunderbad, India); E and F from Neemoil Australia Pty Ltd (Lismore, NSW, Australia) provided by M. B. Isman (University of British Columbia, Vancouver, Canada); and G from Trifolio-M (Germany) (courtesy of K. R. S. Ascher of The Volcani

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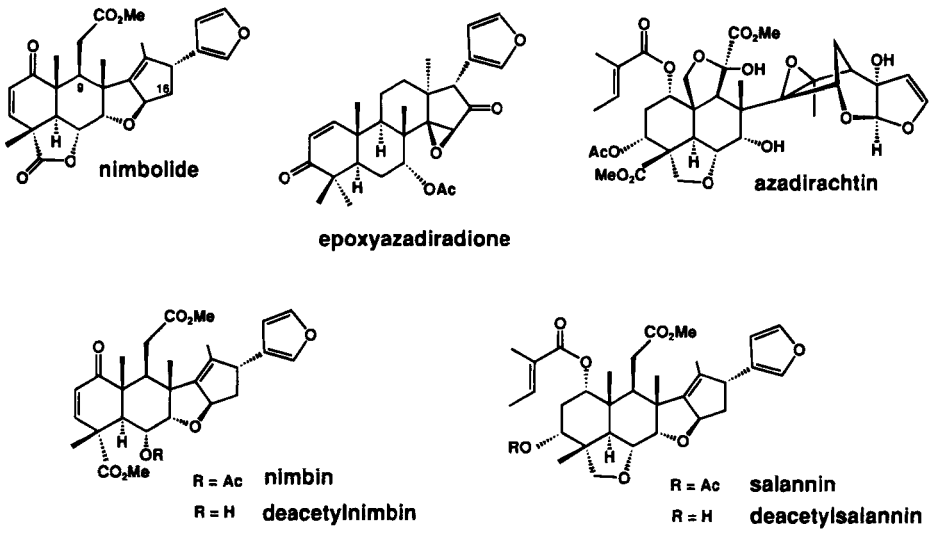

Fig. 1. Structures of azadirachtin and major limonoids in neem extract.

Center, Bet Dagan, Israel). Neem oil from W. R. Grace was also examined. The country refers to the place where the extraction and/or purification was made, not the point of origin since all of the neem seeds for the extracts examined originated from India.

2.2 Cell cultures and viability assays

N1E-115 murine neuroblastoma, 143B.K$^-$ human osteosarcoma and Sf9 *Spodoptera frugiperda* (J. E. Smith) cells were obtained and maintained on media without antibiotics as described elsewhere.[7] The cells, after an initial 24-h incubation in 96-well flat-bottom microplates, were exposed for an additional 24 h to serial dilutions of neem-seed extracts and nimbolide. Stock solutions were prepared for neem extract at 40 mg ml^{-1} in ethanol and from nimbolide at 20 mM in ethanol + acetone (4 + 1 by volume). The stock solutions were diluted at least 100-fold prior to addition to the cultured cells since preliminary experiments revealed that a final concentrations of 1% of the above solvents had no adverse effects on cell morphology and viability. The principal cell viability assay involved the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) test developed by Mosmann[8] with modifications[7] using 96-well plates. As an alternative procedure the cells, grown in 24-well microplates, were assayed by removal of medium, washing once with phosphate-buffered saline (PBS), detaching the cells by vigorous pipetting, and subsequent counting using a Coulter counter. Both cell viability assays involved four replicates per concentration and 3000–4000 cells per replicate. The concentration for 50% inhibition (IC_{50}) was obtained from dose-response curves in which probit cytotoxicity was plotted against log compound concentration.

2.3 HPLC analysis and fractionation of neem extract components

Reversed-phase HPLC for fractionation of neem extract A was performed as follows: Waters 600E solvent delivery system; Waters 994 photodiode array detector at 220 nm; Merck 100RP-18 column (5 μm, 0·4 × 12·5 cm) eluting with 30% (v/v) acetonitrile in water for 2 min followed by linear gradient of 30 to 70% acetonitrile over 18 min at 1·5 ml min^{-1}. In fractionation to identify cytotoxic components, neem extract A (2 mg) was used, collecting 1-min fractions which were evaporated to dryness using a Savant Speed Vac concentrator. These fractions were analyzed for cytotoxicity with N1E-115 cells using the MTT assay. Seven neem extracts and neem oil were analyzed by HPLC using duplicate 50-μg aliquots: Beckman Ultrasphere ODS column (5 μm, 0·46 × 25 cm), 45% (v/v) methanol in water for 2 min followed by linear gradient of 45 to 95% methanol over 28 min at 1·5 ml min^{-1}. The percentage composition for the components was calculated from UV peak areas at 220 nm relative to authentic standards for each compound of known mass. The same HPLC conditions with the Beckman column were used to remove nimbolide from extract A: three 2-mg injections collecting separately nimbolide and all other eluant for 26 min; dilution of eluate fractions with water and extraction of neem components with ethyl acetate; solvent evaporation under vacuum.

2.4 Isolation and identification of limonoids

Nimbin was purchased from Spectrum Chemical Manufacturing Corp. (Gardena, CA). Azadirachtins A and B and deacetylsalannin were provided by M. B. Isman. Initial bioassay-based fractionation of cytotoxic com-

ponents monitored with N1E-115 cells led to the isolation of deacetylnimbin, epoxyazadiradione, nimbolide and salannin from neem extract A (2–8 g) by a three-step procedure: (1) chromatography on a silica gel 60 (0·04 × 0·63 mm) column (2·7 × 37 cm) eluted with hexane + ethyl acetate (120 ml of 2 + 1 and 360 ml of 1 + 1 by volume), collecting 10-ml fractions; (2) TLC on Analtech precoated silica gel GF plates (20 × 20 cm × 1 mm) and (3) HPLC (Merck column and conditions as in Section 2.3). Nimbolide and deacetylnimbin (tubes 32–37 from silica column) separated together from other components after TLC twice (hexane + ethyl acetate, (1 + 1 by volume) $R_f = 0{\cdot}25$, then chloroform + methanol, (150 + 2·3 by volume) $R_f = 0{\cdot}26$). Final purification involved either preparative HPLC on the Merck column (multiple injections of 0·5–1 mg) or TLC twice (ether + hexane, (3 + 1 by volume) nimbolide $R_f = 0{\cdot}25$ and deacetylnimbin $R_f = 0{\cdot}31$). Nimbolide and deacetylnimbin were identified by NMR and fast atom bombardment–mass spectrometry (FAB–MS): for nimbolide (97% purity), [^{1}H] and [^{13}C]NMR spectra agree with literature values[9] except for some couplings of 16-H protons and shift of 9-H observed at δ 2·75 versus quoted at 2·27,[10] $C_{27}H_{31}O_7$ for MH^+ calculated mass = 467·2070, found = 467·2077; for deacetylnimbin (98% purity), [^{1}H] and [^{13}C]NMR in good agreement with published values[9,11] $C_{28}H_{34}O_8Na^+$, calculated mass = 521·2154, found = 521·2150; $C_{28}H_{35}O_8$ for MH^+ = 499 found by low-resolution FAB–MS. Salannin (tubes 40–50 silica column) was purified by TLC (hexane + ethyl acetate, (1 + 1 by volume) $R_f = 0{\cdot}18$): >95% purity; [^{1}H] and [^{13}C]NMR spectra agree with reported values[5,12] except salannin mislabelled as deacetylsalannin for tabulated ^{13}C data.[5] Epoxyazadiradione (tubes 11–26) was also purified by TLC (hexane + ethyl acetate, (2 + 1 by volume) $R_f = 0{\cdot}41$): >95% purity; [^{1}H]NMR signals match literature values;[13] [^{13}C]NMR spectrum consistent with that predicted from benzoate derivative,[14] $C_{28}H_{34}O_6$ for M^+ found at m/z = 466 by electron impact MS.

3 RESULTS

3.1 Cytotoxicity of neem extracts (Figs 2 and 3)

Assay for cytotoxicity of neem extract A using the N1E-115 cell line gave IC_{50} values of 10 and 20 μg ml^{-1} using total cell count and MTT analysis, respectively. Five other neem-seed extracts were cytotoxic to N1E-115 cells with IC_{50} values of 20–200 μg ml^{-1}. Two additional cell lines (143B.TK$^-$ from human osteosarcoma and Sf9 from *Spodoptera frugiperda*) were also sensitive to the toxic effects of these neem extracts, with the insect cells generally more susceptible than the mammalian cells. A seventh neem-seed extract (G), subsequently shown to contain relatively more azadirachtin and less lipophilic limonoids, was significantly less cytotoxic.

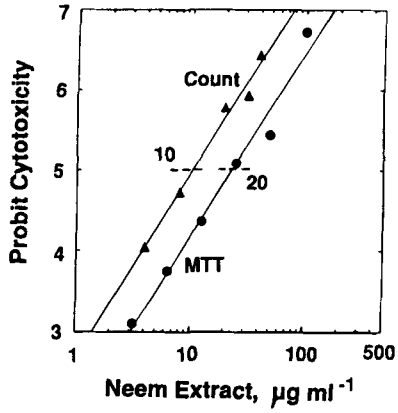

Fig. 2. Cytotoxicity of neem extract A to N1E-115 cells based on (●) MTT assay and (▲) cell count.

3.2 HPLC separation of neem extract A and cytotoxicity of fractions (Fig. 4)

Fractionation of neem extract A by HPLC and bioassay using N1E-115 cells clearly demonstrated the lack of cytotoxicity for azadirachtin, but, instead, several UV-active peaks with variable levels of toxic action. The HPLC peaks with cytotoxicity were purified by a combination of preparative HPLC and TLC to give deacetylnimbin, epoxyazadiradione, nimbolide and salannin which were identified by [^{1}H] and [^{13}C]NMR and MS. Azadirachtin, deacetylsalannin and nimbin were identified by HPLC comparison to authentic standards. The composition of the cytotoxic peaks is indicated in the legend of Fig. 4.

3.3 Composition of limonoids in neem extracts and neem oil (Table 1)

The initial HPLC conditions were inadequate for limonoid analysis, as evidenced by the fact that the most

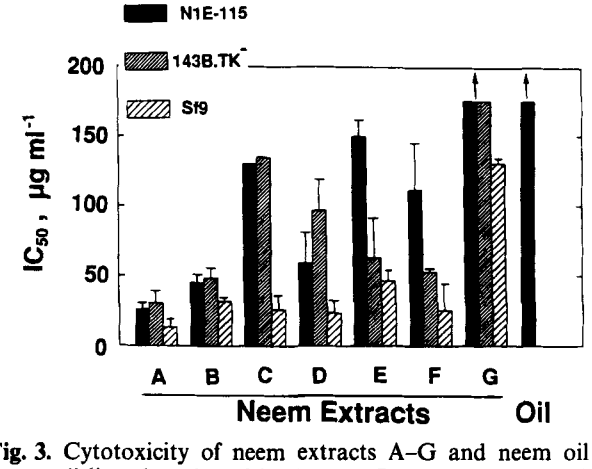

Fig. 3. Cytotoxicity of neem extracts A–G and neem oil to three cell lines based on MTT assay. Bars represent standard deviation for four replicates.

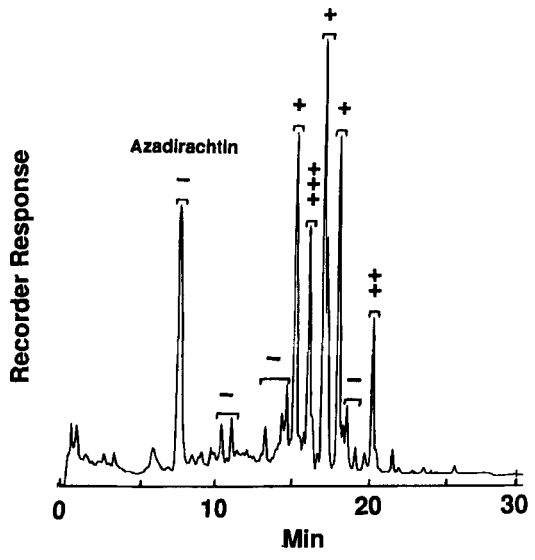

Fig. 4. HPLC separation of neem extract A (Merck column) and cytotoxicity of fractions. The azadirachtin peak is designated. Cytotoxicity is rated as − to +++. The composition of cytotoxic peaks in order of elution was: + deacetylnimbin plus deacetylsalannin; +++ 27% nimbolide and a second major unidentified compound of lower cytotoxicity; + unidentified; + >95% salannin; ++ >95% epoxyazadiradione.

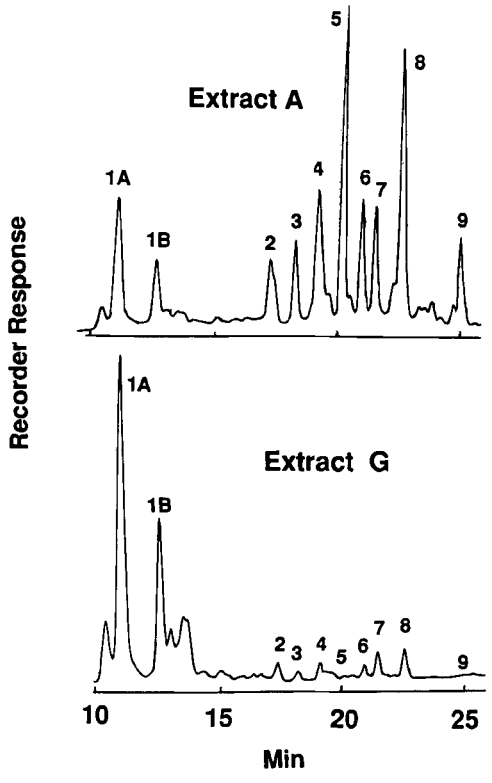

Fig. 5. HPLC separation of neem extracts A and G showing positions of limonoids (Beckman column). 1A azadirachtin A; 1B azadirachtin B; 2 nimbolide; 3 unidentified; 4 deacetylnimbin; 5 unidentified; 6 nimbin; 7 deacetylsalannin; 8 salannin; 9 epoxyazadiradione.

cytotoxic region was only 27% nimbolide (Fig. 4). Modified HPLC conditions (Fig. 5) allowed good resolution of nimbolide and other limonoids for quantification relative to the UV response of authentic standards. Azadirachtin was the major component in all extracts (except F) with salannin the second most abundant constituent. Neem oil contained no detectable azadirachtin and ≦1% as combined limonoids which accounts for its lack of cytotoxicity.

3.4 Nimbolide as a major cytotoxic component

The amount of nimbolide in neem extracts generally correlated with observed cytotoxicity, e.g. the most toxic extract (A) contained 2·3% nimbolide while the

TABLE 1
Composition of Limonoids in Seven Neem Extracts and Neem Oil Analyzed by HPLC

		Limonoid (%)[b]							
		Neem extracts							
Limonoid	*HPLC desig.*[a]	*A*	*B*	*C*	*D*	*E*	*F*	*G*	*Neem oil*
Azadirachtin[c]	1A + B	18·6	15	26·7	36·4	16·4	9·3	30	<0·1
Deacetylnimbin	4	7·8	4·1	1·5	1·1	5·3	7·2	0·8	<0·03
Deacetylsalannin[d]	7	4·1	1·9	0·5	0·4	8·2	9·1	0·9	0·05
Epoxyazadiradione	9	3·2	1·6	0·6	0·4	< 0·2	<0·2	0·1	0·3
Nimbin	6	4·8	2·2	2·5	2·5	2·8	5·9	0·7	0·1
Nimbolide	2	2·3	1·0	0·9	0·8	< 0·2	<0·2	<0·3	<0·03
Salannin	8	10·4	4·6	4·3	4·3	12·3	23·0	1·1	0·2

[a] See Fig. 5.
[b] Percentages (average of duplicate analyses) are maximum values based on HPLC peaks which may contain additional components.
[c] Value from supplier.
[d] Peak area compared with salannin which is assumed to have the same UV response.

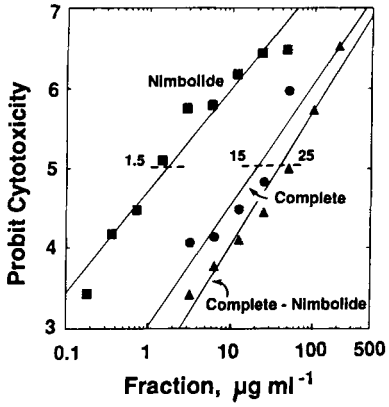

Fig. 6. Cytotoxicity of neem extract A (●) with and (▲) without the nimbolide component. (■) Nimbolide alone. The nimbolide fraction was removed from the reconstituted components following HPLC separation. Each point represents an average of eight replicates.

least active (G) contained <0·3% (Table 1, Figs 3 and 5). Nimbolide is the most toxic component in neem extract for neuroblastoma cells (IC_{50} 1·5 μg ml^{-1}, Fig. 6; equivalent to 3·2 μM). While the complete extract has an IC_{50} of 15 μg ml^{-1}, removal of nimbolide increases the IC_{50} to 25 μg ml^{-1} (Fig. 6).

4 DISCUSSION

Neem-seed extracts utilized for insecticide preparations have variable cytotoxicities to the mammalian and insect cultured cells assayed. Azadirachtin, nimbin and deacetylnimbin do not appear to contribute to the cytotoxicity based on this study and assay of the individual limonoids.[7] Some of the cytotoxicity is due to epoxyazadiradione, salannin and possibly deacetylsalannin, which are cytotoxic and sometimes appear in relatively large amounts. The major portion of toxicity to neuroblastoma cells is attributable to nimbolide on the basis of its relative potency and amount and because the IC_{50} of neem extract A is increased from 15 to 25 μg ml^{-1} on removal of the 2% nimbolide component. The cytotoxic action for nimbolide involves rapid blebbing associated with membrane disruption for N1E-115 cells as detailed elsewhere.[7] The neem oil studied here lacks nimbolide and has only low levels of other limonoids, but since 10–20-fold more neem oil is used relative to azadirachtin in certain insecticide formulations,[15] the total limonoid contribution from neem oil and its associated toxicity can be substantial.

Nimbolide is moderately toxic to bacteria, cultured cells and mammals (Table 2). Although not mutagenic in *Salmonella typhimurium* Castell. & Chalm. with or without metabolic activation,[16,17] it is toxic to *Staphylococcus*.[16] Nimbolide is moderately toxic to *Plasmodium falciparum* in culture, but its possible use as an antimalarial agent is contra-indicated by poor effectiveness against a related species in mice *in vivo*.[18,19] It is moderately toxic (IC_{50} = 0·1–10 μM) to cell lines in culture from eight human tumors, murine neuroblastoma and an insect.[7,10] In contrast to sodium nimbinate,[21] nimbolide has little effect on mammalian sperm.[7] Although nimbolide is toxic to mammals when administered intravenously or intraperitoneally, it is considerably less active orally.[20] Mammalian toxicity is

TABLE 2
Biological Activity of Nimbolide

System	*Activity*	*Reference*
Bacteria	Not mutagenic at 0·5 mg per filter disc in *Salmonella typhimurium* using six tester stains (with or without S-9 activation), but toxic to three species of *Staphylococcus* under similar conditions	16, 17
Malaria	Inhibits *Plasmodium falciparum in vitro* at 1 μg ml^{-1} but not *P. berghei* in mice *in vivo* either by ingestion or subcutaneous injection	18, 19
Mammalian and insect cultured cells	Cytotoxic (IC_{50} = 0·1–1·1 μM) for seven human tumor cell lines (lymphocytic leukemia, nasopharynx carcinoma, human fibrosarcoma, lung, colon, melanoma, breast); cytotoxic (IC_{50} = 4–10 μM) for murine neuroblastoma, human osteosarcoma and Sf9 insect cells	7, 10
Sperm	20–30% reduced in rabbit sperm motility and viability on exposure at 50 μM for 60 min	7
Rodents	Mouse LD_{50} > 600 mg kg^{-1} oral, 225–280 mg kg^{-1} i.p. and 24 mg kg^{-1} i.v.; rat and hamster LD_{50} > 500 mg kg^{-1} i.p.; toxic dosage i.p. causes necrosis in kidney, liver, intestine and pancreas and i.v. causes sudden drop in blood pressure and respiratory paralysis with death within 1–18 min from hypotensive shock	20

accompanied by necrosis in a variety of tissues[20] consistent with the finding of general cytotoxicity for cell lines from very different sources.[7]

Although the effectiveness of neem extracts depends on the azadirachtin content, their safety may be related in part to minor constituents. The use of azadirachtin-containing neem extracts for pest insect control requires an understanding of the biological activity of the other major constituents many of which are limonoids. The nimbolide content can be reduced by selecting sources low in this limonoid or by partial removal during processing.

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